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### Gene delivery with cationic lipids

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## **Lipoplexes formed from sugar-based gemini surfactants undergo a lamellar-to-micellar phase transition at acidic pH. Evidence for a non-inverted membrane-destabilizing hexagonal phase of lipoplexes**

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### Abstract

The present study aims at a better understanding of the mechanism of transfection mediated by two sugar-based gemini surfactants GS1 and GS2. Previously, these gemini surfactants have been shown to be efficient gene vectors for transfection both in vitro and in vivo. Here, using Nile Red, a solvatochromic fluorescent probe, we investigated the phase behavior of these gemini surfactants in complexes with plasmid DNA, so-called lipoplexes. We found that these lipoplexes undergo a lamellar-to-non-inverted micellar phase transition upon decreasing the pH from neutral to mildly acidic. This normal (non-inverted) phase at acidic pH is confirmed by the colloidal stability of the lipoplexes as shown by turbidity measurements. We therefore propose a normal hexagonal phase,  $H_I$ , for the gemini surfactant lipoplexes at acidic endosomal pH. Thus, we suggest that besides an inverted hexagonal ( $H_{II}$ ) phase as reported for several transfection-potent cationic lipid systems, another type of non-inverted non-bilayer structure, different from  $H_{II}$ , may destabilize the endosomal membrane, necessary for cytosolic DNA delivery and ultimately, cellular transfection.

Keywords: cationic lipid, gemini surfactant, pH sensitive, transfection mechanism, hexagonal phase, Nile Red fluorescence, endosomal release.

## **Introduction**

Because of their low immunogenicity, their relative ease of production and chemical modification, cationic lipids are considered a promising alternative to viral vectors for the cellular delivery of genes *in vitro* and *in vivo*. However, a detailed understanding of the mechanisms by which cationic lipids can mediate transfection is of primary importance in order to optimize gene delivery *per se* and to improve their versatility for *in vivo* applications, which are still inferior to those obtained for viral vectors. [1,2].

The release of plasmid DNA from the endosomal compartment is a key step in the mechanism of transfection, mediated by cationic lipids. However, the mechanism by which plasmid DNA can escape from endosomes is still poorly understood. Presumably, lipoplexes enter the cells via endocytosis and a subsequent destabilization of the endosomal membrane, accompanied by a dissociation of the gene from the carrier, is necessary to allow the release of the cargo DNA into the cytoplasm [3]. Using small angle X-ray scattering (SAXS) measurements and optical microscopy, Koltover et al. demonstrated that the helper lipid DOPE induces the formation of an inverted hexagonal ( $H_{II}$ ) phase in the widely-used cationic lipid-based delivery system DOTAP/DOPE that strongly promotes DNA release [4]. The same  $H_{II}$  phase was demonstrated to occur in SAINT-2/DOPE mixtures at high salt concentrations as demonstrated by NMR-spectroscopy, cryo-transmission electron microscopy and SAXS, and evidence was provided that supported the conclusion that  $H_{II}$  formation is a prerequisite for effective release of DNA and oligonucleotides from endosomes [5-7].

Depending on the molecular nature of the cationic lipid, the formation of such non-bilayer structures may be promoted by the presence of non-bilayer-phase promoting helper lipids like DOPE, and may therefore be pre-existing, which may cause extensive clustering of lipoplexes. However, non-bilayer structures can also be triggered and/or promoted upon interaction of SAINT-2 containing lipoplexes with phosphatidylserine (PS)-containing lipid vesicles [8], in line with a proposal that PS translocation across endosomal membranes is instrumental in the mechanism of lipoplex-mediated transfection [9]. Further support for this notion was obtained in studies, using the EDLPC/EDOPC system, in which it was shown that DNA dissociation and transfection efficiency correlate with the propensity of cationic/anionic lipid mixtures to evolve into highly curved mesomorphic structures, i.e. inverted hexagonal or inverted micellar cubic phases [10,11]. Interestingly, besides an inverted  $H_{II}$  phase, another type of non-inverted hexagonal structures ( $H_I$ ) has been reported

for the single-tailed surfactant CTAB [12,13]. X-ray diffraction studies showed that this amphiphile in complexes with DNA forms a 2D hexagonal lattice. Concomitant addition of sodium 3-hydroxy-2-naphthoate (SHN), which decreases the spontaneous curvature of CTAB micelles, has been shown to promote the hexagonal to lamellar transition of CTAB lipoplexes, thus revealing that CTAB is arranged in a non-inverted phase [12].

The sugar-based gemini surfactants used in the present study, have been shown to display a lamellar phase at neutral pH, whereas a micellar phase can be triggered, in the absence of any helper lipid, by a mildly acid pH, as occurs in early endosomes [14-16]. This system thus offers the obvious advantage that little particle clustering takes place upon lipoplex assembly and when injected into the circulation in vivo [17] and the potential of such systems for gene delivery in vitro and in vivo has been demonstrated [17-19].

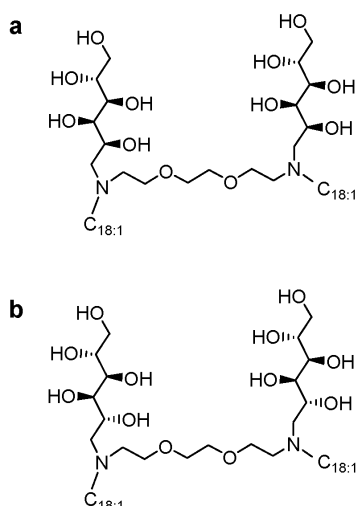
SAXS measurements and cryo-electron microscopy of lipoplexes prepared from one of these gemini surfactants (GS1) showed the formation of a hexagonal phase at mildly acidic pH, and it was postulated that the DNA could serve as a template for an inverted  $H_{II}$  columnar phase [19].

However, additional work, presented here, led us to propose a different mechanism of action and endosomal release mediated by these sugar-based gemini surfactants. Our data support a non-inverted micellar organization of the gemini lipoplexes rather than an inverted hexagonal organization, as suggested previously. Using a Nile Red-based assay [20], the phase behavior of two transfection-potent gemini surfactants was investigated and compared to that of SAINT-2/DOPE, a classical  $H_{II}$  phase forming system. We demonstrate that these gemini lipoplexes undergo a lamellar-to-micellar phase transition in the endosomal pH range.

## **Experimental section**

### ***Materials***

The sugar-based gemini surfactants GS1 and GS2 (Fig. 1) were synthesized as previously described [14,15]. The plasmid DNA used was pEGFP-N1 from Clontech laboratories. The plasmid was propagated in *Escherichia coli* strains and DNA was extracted using a genelute plasmid midi-prep kit from Sigma. Nile Red was obtained from ACROS (Landsmeer, The Netherlands). N-NBD-phosphatidylethanolamine (N-NBD-PE), N-Rhodamin-phosphatidylethanolamine (N-RH-PE), dioleoylphosphatidylethanolamine (PE), dioleoylphosphatidylcholine (PC) and dioleoylphosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).



**Figure 1. Chemical structures of GS1 (a) and GS2 (b).** The basic structures of the sugar-based gemini surfactants GS1 and GS2 consist of two single-tail surfactants with a reduced sugar and a pH sensitive amino moiety in the headgroup and an oleyl chain as hydrocarbon tail. Both twin structures are linked via an ethylene oxide spacer between the two tertiary nitrogens. GS1 (a) and GS2 (b) differ by the nature of the reduced sugar, glucose and mannose respectively. Note that the degree of protonation of the two nitrogens within the sugar-based gemini surfactant is dependent on pH. Consequently, these amphiphiles are fully charged at mildly acidic pH.

### Nile Red assay

The use of Nile Red as a probe for determining the phase behavior of surfactant aggregates has been described by Stuart et al. [20]. In essence, this assay monitors a shift in the emission maximum of the Nile Red probe, integrated into the lipid phase of lipoplexes. Thus relative to its emission maximum when present in the lamellar phase, this maximum shifts to longer wavelengths (more polar) when the system transfers to a micellar phase and to shorter wavelengths (more hydrophobic), when the lamellar phase converts into an inverted micellar phase.

The procedure is carried out as follows. Briefly, a 2.5 mM Nile Red stock solution was made in ethanol and diluted 2500-fold in the surfactant systems. Nile Red fluorescence was measured on an SPF-500c spectrofluorimeter (SLM Aminco) at 25°C. The excitation wavelength was set at 550 nm and the fluorescence emission was recorded from 550 to 700 nm at 5 nm intervals. The wavelength of the emission maximum ( $\lambda_{\text{max}}$  emission) of Nile Red was calculated using a 4 parameters log-normal fit. Vesicles of the different sugar-based gemini surfactants were prepared at pH 6.7 in a 5 mM MES/HEPES/sodium acetate buffer at a 0.1 mM final concentration and in a final volume of 4 ml. The lipids (fixed amount of 0.4  $\mu\text{moles}$ ) were then freeze/thawed 5 times, after which plasmid DNA was added in amounts corresponding to different charge molar ratios (+/-), taking into account that one molecule of gemini surfactant carries on average one positive charge. The charge ratios investigated were 8:1, 2:1 and 1:2, corresponding to 16  $\mu\text{g}$ , 64  $\mu\text{g}$  and 256  $\mu\text{g}$  of plasmid DNA, respectively. The Nile Red emission maximum was determined at different pH values, using a protocol in

which the pH was first lowered step by step to acidic pH (approximately pH 3), then raised to pH 6.7, and subsequently increased step by step from pH 6.7 to approximately pH 8.5.

### ***Turbidity measurements***

The turbidity of lipoplex dispersions, providing a measure of their colloidal stability, was monitored as a function of time on a Perkin Elmer LAMBDA 25 UV/Vis spectrometer at a wavelength of 350 nm. The final concentration of sugar-based gemini surfactants was 0.1 mM and the (+/-) charge molar ratio was 8 to 1. The colloidal stability was compared to that of SAINT-2/DOPE (1:1) lipoplexes at a 2.5 to 1 charge molar ratio (+/-) [5]. Three different conditions were tested: in 5 mM MES/HEPES/sodium acetate buffer at pH 7 or in salt solution (HBS: 10 mM HEPES, 150 mM NaCl) at pH 5 or 7. The turbidity values reached after 15 min of incubation are represented in the graphs (average values from 15 to 20 min).

### ***Lipid mixing assay***

Lipid mixing was monitored by an assay based on resonance energy transfer between two lipid probes as described earlier [21,22]. GS1 liposomes containing 0.5 % N-NBD-phosphatidylethanolamine (N-NBD-PE) and 0.5 % of N-Rhodamin-phosphatidylethanolamine (N-RH-PE) were prepared at pH 6.7 in MES/HEPES/sodium acetate buffer (5 mM) at 1 mM as described above. Lipoplexes were prepared with 0.1  $\mu$ mole of lipid, mixed with 4  $\mu$ g of plasmid DNA (charge molar ratio 8:1) and diluted in MES/HEPES/sodium acetate buffer at pH 6, pH 7 and pH 8 (as indicated) at a final lipid concentration of 0.1 mM. Fluorescence development, occurring upon relief of energy transfer, was monitored for 500 s on a LS-55 spectrofluorimeter (PerkinElmer); the emission and excitation wavelengths were set to 450 and 530 nm respectively. PE/PC/PS (2:1:1) vesicles or PC/PE (1:1) vesicles, as indicated, were added after 100 s at a 5 fold excess of lipids, compared to the lipoplexes. The maximum level of fluorescence was obtained by adding Triton X-100 detergent after 250 s at a final concentration of 0.2%. The percentage of lipid mixing was calculated as follows:

$$(1) \% \text{ Lipid Mixing} = \frac{F - F_0}{F_{100} - F_0} \times 100$$

$F_0$  is the initial fluorescence intensity;  $F$  is the fluorescence intensity obtained following interaction of the lipoplexes with either PE/PC/PS or PE/PC vesicles;  $F_{100}$  is the maximum fluorescence intensity reached upon infinite dilution, obtained after addition of

Triton X-100. All values reported were corrected (if necessary) for detergent quenching and sample dilution

***Plasmid release assay***

Plasmid release was monitored by a PicoGreen assay (Molecular Probes). Lipoplexes were prepared as described for the lipid mixing assay and diluted in a PicoGreen containing MES/HEPES/sodium acetate buffer at pH 6 and pH 8 at a final concentration of 0.1 mM. The fluorescence of PicoGreen, directly proportional to the presence of accessible plasmid DNA, was monitored on a LS-55 spectrofluorimeter; the emission and excitation wavelengths were set to 485 and 520 nm respectively. PE/PC/PS (2:1:1) vesicles or PC/PE (1:1) vesicles, as indicated, were added after 100 s in a 5-fold excess over the lipoplexes. The maximum fluorescence was obtained by adding Triton X-100 at a final concentration of 0.2 %. The percentage of plasmid DNA release is expressed as follows:

$$(2) \% \text{ Plasmid Release} = \frac{F - F_0}{F_{100} - F_0} \times 100$$

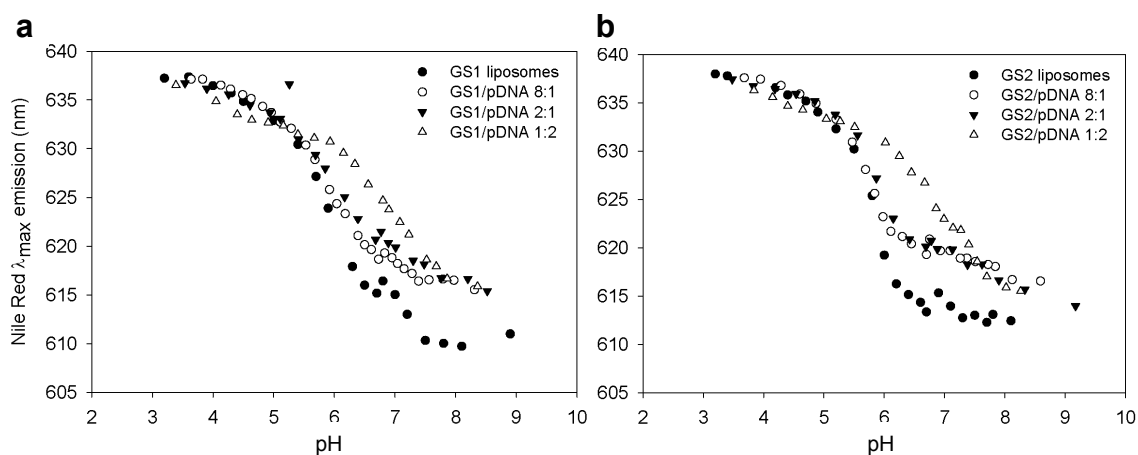
$F_0$  is the initial fluorescence,  $F$  is the fluorescence of the sample after addition of PE/PC/PS or PE/PC vesicles and  $F_{100}$  is the maximum fluorescence corrected for the increase due to the effect of the detergent (as specified by Molecular Probes).

**Results*****Phase transition of GS1 and GS2 lipoplexes upon acidification***

The phase behavior of GS1 and GS2 liposomes and lipoplexes, at molar charge ratios (+/-) of 8:1, 2:1 and 1:2, was investigated as a function of pH, employing the Nile Red assay as described in the Experimental section. Figure 2a and 2b summarize the results for GS1 and GS2, respectively. The  $\lambda_{\text{max}}$  emission of Nile Red in pure GS1 aggregates was around 610 nm at pH values between pH 8 and 9 and increased to 637 nm between pH 3 and 4. Similarly, in pure GS2 aggregates the  $\lambda_{\text{max}}$  emission of Nile Red increased from 613 nm between pH 7 and 8 to 638 nm between pH 3 and 4. This reflects a vesicle-to-micelle transition of the pure lipids, induced by the lower packing parameter at acidic pH [14]. The phase behavior of GS1 lipoplexes as a function of pH at an 8 to 1 molar charge ratio (+/-) followed the same trends as the pure lipids (Fig. 2a GS1/pDNA 8:1). The plasmid DNA induced a higher  $\lambda_{\text{max}}$  emission at basic pH compared to the pure lipids, with a value of 615 nm above pH 8. This difference most likely reflects an effect of DNA binding to the cationic lipid on the polar



environment of the Nile Red probe in a lamellar phase. At more acidic pH values the  $\lambda_{\max}$  emission increases and reaches an average value of 637 nm between pH 3 and 4. A similar trend was seen for GS2 lipoplexes of which, at an 8 to 1 (+/-) molar charge ratio,  $\lambda_{\max}$  emission increases from approximately 617 nm to 638 nm upon acidification. It should be noted that at a molar charge ratio (+/-) of 8 to 1, GS1 and GS2 lipoplexes show optimal transfection efficiency, as reported elsewhere [17]. Evidently, at this charge ratio the lipids are in excess over plasmid DNA and, consequently, the phase behavior described with Nile Red might partly reflect a contribution of free lipids that are not bound in complexes with DNA. In order to rule out this possibility, Nile Red assays were also performed with higher and excess amounts of plasmid DNA at a molar charge ratio (+/-) of 2:1 and 1:2, respectively (Fig. 2). In these cases, the  $\lambda_{\max}$  emission of Nile Red for both GS1 and GS2 lipoplexes increased from approx. 615 nm at alkaline pH to 637 nm at acidic pH. Interestingly, upon acidification the transition from a lamellar to a micellar phase seemed to occur more readily for lipoplexes than for liposomes. Thus, for GS1 liposomes  $\lambda_{\max}$  emission of Nile Red at pH 7.0 was 614 nm while for the lipoplexes, irrespective of the charge ratio, this value centered around 620 nm. Likewise for GS2 liposomes the  $\lambda_{\max}$  emission of Nile Red at pH 7.1 is 615 nm, whereas for lipoplexes at similar pH conditions this value is around 622 nm. Taken together, these results thus indicate that GS1 and GS2 lipoplexes, similarly to the liposomes, undergo a bilayer-to-micellar transition at acidic pH values, while the presence of plasmid DNA apparently promotes this transition.

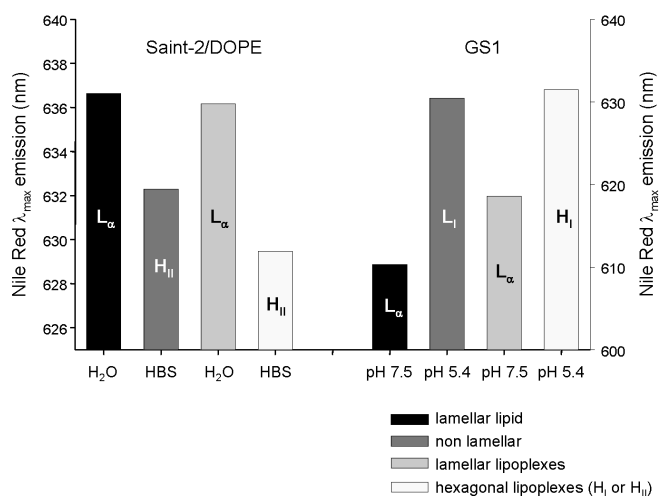


**Figure 2. Lamellar-to-micellar phase transition of sugar-based gemini liposomes and lipoplexes upon acidification.** The maximum emission wavelengths ( $\lambda_{\max}$  emission) of Nile Red in GS1 (a) and GS2 (b) liposomes and lipoplexes were determined as a function of pH. In complexes with plasmid DNA, molar charge ratios (+/-) of 8:1 2:1 and 1:2 were tested (GS1/pDNA and GS2/pDNA 8:1; 2:1 and 1:2, respectively). Note that upon acidification the  $\lambda_{\max}$  emission of Nile Red increases consistent with its exposure to a more polar microenvironment and the conversion of the gemini lipoplexes to a normal micellar phase.

***GS1 and GS2 lipoplexes compared with lipoplexes of known morphology***

In order to distinguish between an inverted and a normal phase of the cationic lipid in GS1 liposomes and lipoplexes, the phase properties of these systems, as determined with the Nile Red assay, were compared to well-documented phase behavior of the gene delivery vector SAINT-2/DOPE (1:1). In water, SAINT-2/DOPE lipoplexes and liposomes exhibit a lamellar  $L_{\alpha}$  organization, while at physiological salt concentrations they adopt an inverted hexagonal  $H_{II}$  phase [5,7]. This propensity was then exploited to verify and validate the behavior of Nile Red in distinguishing the inverted hexagonal phase from the lamellar phase, reflected by a hypsochromic shift in emission maximum. The results, shown in Fig. 3, indicate that in water the  $\lambda_{\max}$  emission of Nile Red in SAINT-2/DOPE liposomes is 636 nm while in a physiological salt solution (HBS) the  $\lambda_{\max}$  emission is 632 nm. For SAINT-2/DOPE lipoplexes the measurements gave a  $\lambda_{\max}$  emission of 636 nm in water, and a value of 629 nm was obtained for lipoplexes, suspended in HBS (Fig. 3), i.e. conditions at which the lipoplexes display an inverted  $H_{II}$  phase. Accordingly, these data indicate that in an inverted hexagonal phase,  $\lambda_{\max}$  emission of Nile Red is lower than that obtained for the lamellar phase. For comparison, the Nile Red emission maxima are included in Fig. 3, obtained for GS1 liposomes and lipoplexes at pH values representative of the pH range to which lipoplexes are exposed when entering the early endosomal pathway (pH 7.5 and pH 5.4). Consistent with the data in Fig. 2, the  $\lambda_{\max}$  emission measured at pH 7.5 and pH 5.4 for pure GS1 was 610 nm and 630 nm, respectively. Together these data demonstrate that lipoplexes undergoing a transition from a lamellar to a hexagonal  $H_{II}$  phase (SAINT-2/DOPE) show a decrease in  $\lambda_{\max}$  emission of Nile Red, as opposed to a bathochromic shift as seen for GS1 lipoplexes, facing an endosomal pH environment. For GS1 lipoplexes a transition from a lamellar to an inverted micellar structure can thus be excluded, and the data rather support a non-inverted micellar structure, in line with the established correlation between fluorescent and structural properties, detected with this assay (see Experimental section; [20]).

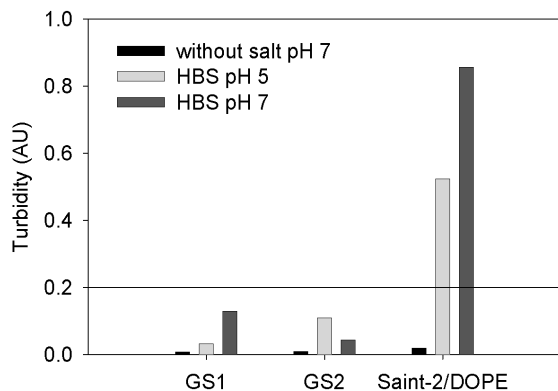
To obtain further support for the notion that GS1 and GS2 lipoplexes may adopt a non-inverted phase, we next investigated the colloidal stability of the complexes, taking into account that those adopting the  $H_{II}$  phase, readily aggregate [6].



**Figure 3. Phase transition of SAINT-2/DOPE and GS1 lipoplexes as monitored by Nile Red.** The  $\lambda_{\max}$  emission of Nile Red in GS1 lipoplexes at pH 7.5 and 5.4 was compared to that in SAINT-2/DOPE lipoplexes in water (H<sub>2</sub>O) and in salt (HBS). Note that a transition from lamellar L <sub>$\alpha$</sub>  to inverted hexagonal H<sub>II</sub> phase, as occurs for SAINT-2/DOPE lipoplexes, translates into a decrease in  $\lambda_{\max}$  emission. In contrast, a transition from a lamellar L <sub>$\alpha$</sub>  to a normal hexagonal H<sub>I</sub> phase translates into an increase in  $\lambda_{\max}$  emission.

### Colloidal stability of GS1 and GS2 lipoplexes

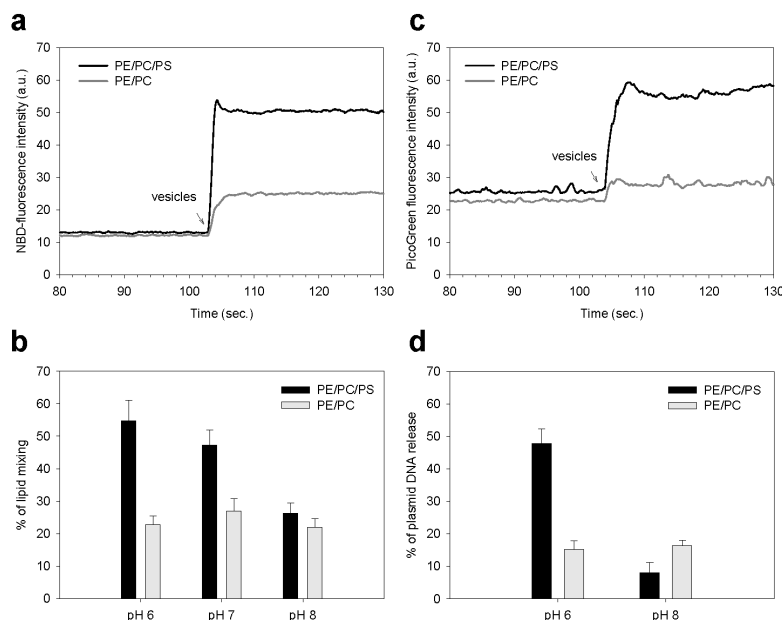
The colloidal stability of GS1, GS2 and SAINT-2/DOPE lipoplexes was studied by turbidity measurements. The turbidity of the lipoplexes reached after 15 min is presented in Fig. 4. For SAINT-2/DOPE in salt free buffer at pH 7 the turbidity stayed at a low level of around 0.02 (arbitrary unit), which was maintained for periods up to at least 24 h. By contrast, in the presence of salt, either at pH 5 or pH 7, the turbidity of these lipoplexes increased rapidly and after 20 min an almost 25-50 fold increase in turbidity was seen. These observations emphasize that lipoplexes, which display a lamellar phase, are colloiddally stable, whereas conversion to an inverted hexagonal H<sub>II</sub> phase, as is the case for SAINT-2/DOPE in salt, causes rapid aggregation and precipitation. Interestingly, as shown in Fig. 4, the aggregation behavior of the lipoplexes prepared from GS1 and GS2 was quite different and, at all relevant conditions, such as in the presence or absence of salt and either at mildly acidic (‘endosomal’) pH or at neutral pH, no significant clustering of the lipoplexes could be detected. Even after 24 hours no precipitates were observed. These results thus suggest that GS1 and GS2 lipoplexes are colloiddally stable, under conditions where SAINT-2/DOPE lipoplexes adopt an H<sub>II</sub> phase and show extensive clustering. Accordingly, these data for GS1 and GS2 would be consistent with a lamellar phase at pH 7 and a normal micellar phase at pH 5.



**Figure 4: Effect of pH and salt on the colloidal stability of GS1, GS2 and SAINT-2/DOPE lipoplexes.** The turbidity of GS1, GS2 and SAINT-2/DOPE lipoplexes was monitored at 350 nm as a function of time. The histogram presents the average turbidity value (arbitrary unit) reached after 15 min. As indicated in the figure, lipoplex stability was determined at three different conditions, i.e., in buffer without salt at pH 7, in HBS at either pH 5 or pH 7. Note that in salt solutions (HBS) the hydrophobic,  $H_{II}$ -forming SAINT-2/DOPE lipoplexes aggregate as reflected by the increase in turbidity. Under all conditions the turbidity of gemini lipoplexes remains low, consistent with a lamellar  $L_{\alpha}$  organization at pH 7 and a normal micellar phase at pH 5, irrespective of the presence of salt.

### *pH-dependent interaction of GS1 lipoplexes with target membranes; role of PS*

To further corroborate the pH-dependent destabilization properties of these amphiphiles, we next investigated the effect of pH on the interaction of GS1 lipoplexes with lipid vesicles, taking into account that lipid mixing in particular may reflect such a destabilization process. In addition, in the context of a facilitating role of endosomal membrane localized phosphatidylserine (PS) in this process [9], including its role in causing DNA release, we thus examined the interaction of GS1 lipoplexes with PE/PC/PS (2:1:1) and PE/PC (1:1) vesicles. A lipid mixing assay, based on resonance energy transfer was used as described in the Experimental section. As shown in Fig. 5a, at pH 6.0 extensive lipid mixing occurs, as reflected by the almost instantaneous increase in NBD fluorescence, when PS-containing lipid vesicles were incubated with GS1 lipoplexes, whereas a much slower and lesser degree of mixing was apparent when PS was omitted from the target membrane vesicles. As summarized in Fig. 5b, lipid mixing was particularly prominent at pH values faced by the lipoplex when residing in endosomal compartments. As shown in Fig. 5c, when monitoring DNA accessibility by monitoring the development of PicoGreen fluorescence, occurring when this probe associates with either exposed or released DNA, the fluorescence similarly increased when the lipoplexes were interacting at mild acidic pH with PS-containing lipid vesicles, little release being apparent at elevated pH or with vesicles devoid of PS (Fig. 5d). Accordingly, these data are consistent with a pH-dependent capacity of the gemini GS1 lipoplexes to cause membrane destabilization and presumably DNA release, particularly at conditions that match the endosomal environment.



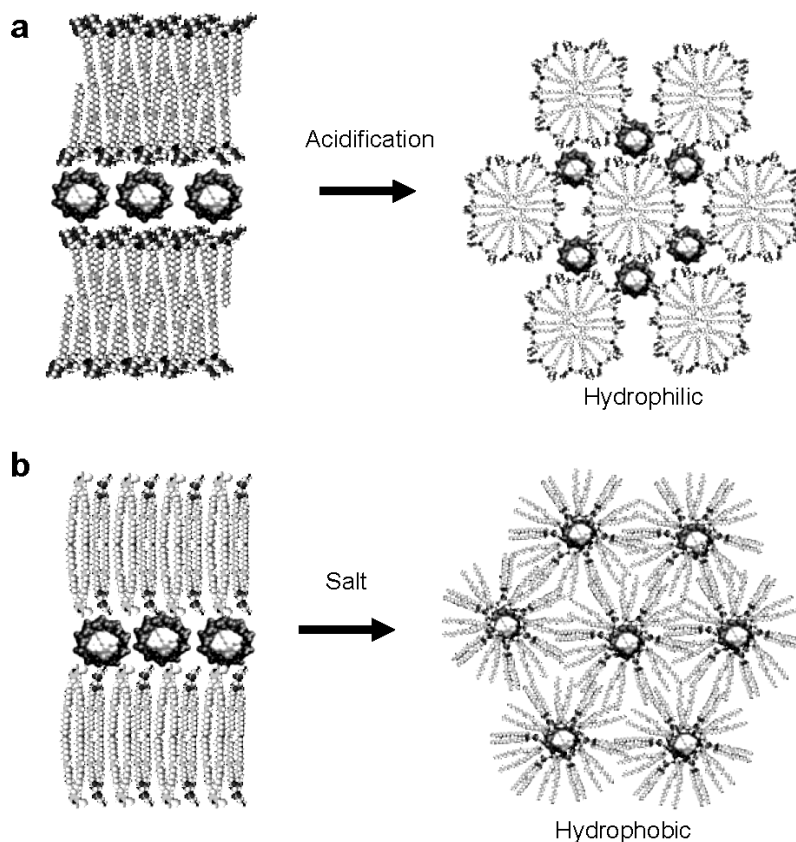
**Figure 5: pH dependence of lipid mixing and PS-mediated DNA release upon GS1 lipoplex-lipid vesicles interaction.** In (a) lipid vesicles, consisting of either PE/PC/PS (2:1:1) or PE/PC (1:1), were mixed with N-NBD-PE/N-RH-PE-labeled GS1 lipoplexes and lipid mixing was monitored by an increase of NBD-fluorescence, occurring upon relief of energy transfer. The interaction was monitored at room temperature at pH 6.0, as described in the Experimental section. (b) From traces as those obtained in a, the percentage of lipid mixing at pH 6, 7 and 8 were calculated (equation 1 in the Experimental section). In (c), the development of PicoGreen fluorescence, reflecting the accessibility of DNA upon interaction of the lipoplexes with the lipid vesicles, composed as described in a, was monitored at pH 6.0. (d) The percentage of DNA accessibility towards PicoGreen was calculated from traces like those shown in c (using equation 2, Experimental section) and the data obtained at pH 6.0 and pH 8.0, are summarized.

## Discussion

In the present work we have shown that lipoplexes made from sugar-based gemini surfactants GS1 and GS2 undergo a lamellar-to-non-inverted micellar phase transition at acidic pH. In contrast, previous work, based on SAXS measurements, led us to conclude that GS1 lipoplexes convert from a lamellar to an inverted hexagonal  $H_{II}$  phase at mildly acidic conditions [19]. It appears that this interpretation was likely biased by the general and widely accepted concept that in complexes with DNA a hexagonal structure formed with a cationic lipid commonly involves an inverted hexagonal phase. Even more so, this inverted hexagonal phase was further rationalized by observations of efficient transfection mediated by these lipoplexes, which often correlates well with the ability to form an inverted hexagonal ‘lipoplex’ phase [4]. The argument for such an inverted structure of the lipoplexes, even though the pure lipids clearly aggregate in micelles at acidic pH, is that the lipids will arrange themselves around the DNA, acting as a backbone, creating an inverted hexagonal phase. Such a model is reasonable since double-tailed surfactants with a high packing

parameter and especially when used in combination with DOPE as helper lipid, favor negative curvature. However, the results of this study, in which we have used a novel and most sensitive assay to monitor both normal and inverted micellar transitions, show that at acidic pH a normal phase is formed for gemini lipoplexes as opposed to an inverted phase for SAINT-2/DOPE lipoplexes. Accordingly, in conjunction with the observed colloidal stability differences between both types of lipoplexes, the data strongly support the notion that the hexagonal structure found for the GS1 and GS2 lipoplexes must be a normal hexagonal  $H_I$  phase. Whether these normal structures form strictly ordered hexagonal superstructures is not entirely clear yet, since the second- and third-order peaks of the SAXS profile of GS1 lipoplexes at acidic pH, as reported previously [19], are somewhat weak. However, two pieces of evidence would nevertheless plead for a hexagonal phase, when carefully analyzing the previous data. Firstly, the SAXS experiments in this previous study were not presented on a logarithmic scale. If so, the second ordered peak is much more pronounced and hence makes a more convincing case of a hexagonal phase. Secondly, and more importantly, the hexagonal structure was confirmed by the cryo-TEM pictures where Fourier transforms of these images (Figs. 2d, e and inset) clearly reveal the hexagonal pattern [19]. Nevertheless, the appearance of an  $H_I$  phase is not unprecedented for these systems since it has also been reported for lipoplexes formed from the single-tailed surfactant CTAB [12,13,23]. In the study by Krishnaswamy et al. [12], SAXS diffractions patterns were compared of CTAB lipoplexes and lipoplexes that also contained the hydrotrope SHN, which is known to decrease the spontaneous curvature of CTAB micelles. Indeed, the fact that the concentration of SHN needed to provoke the (normal) micellar-to-lamellar phase transition of CTAB micelles is the same as the concentration needed for converting hexagonal CTAB-DNA lipoplexes into lamellar lipoplexes suggests that the lipids in the lipoplexes display the same morphology as the micelles of the pure lipids. Furthermore, Zhou et al. [24] suggest two possible structural models for the 2D hexagonal column phase in CTAB-DNA complexes, as inferred from SAXS measurements. These involve a normal  $H_I$  and an inverted  $H_{II}$  phase. However, the authors argue in favor of the inverted  $H_{II}$  phase as the predominant structure because of the lower intensity of the second-order scattering peak in the SAXS profile, which is more compatible with an inverted phase. Another interpretation could be that if the ordered level of the hexagonal structure is low this will also lead to a second- and third-order scattering peak of relatively lower intensity, implying that an exclusion of a normal phase is as yet premature.

It is not unreasonable to compare the phase behavior of gemini surfactants at acidic pH with that of single-tailed surfactants, since their packing parameter is lower than that at higher pH values [14,15]. The lipids in the gemini lipoplexes behave like the lipids in the liposomal membranes showing that at acidic pH a normal phase is formed. In addition, in the case of sugar-based gemini surfactants, plasmid DNA favors the formation of normal structures (Fig. 2), as inferred from the observation of an earlier bathochromic shift of the emission maximum of Nile Red fluorescence in lipoplexes than in liposomes. By contrast, if DNA would have promoted the formation of the inverted  $H_{II}$  phase a delay in the transition to higher polar aggregates should have been found or no transition at all. Much to the contrary it is observed that in the presence of plasmid DNA, the transition to a more polar phase occurs at higher pH values, implying that the plasmid DNA promotes the transition from a lamellar phase to normal micelles.



**Figure 6: Model for the phase transition of lipoplexes formed from sugar-based gemini surfactants (a); comparison with SAINT-2/DOPE (b).** The model depicted here for the gemini lipoplexes (a) illustrates a transition from a lamellar phase  $L_\alpha$  to a normal hexagonal  $H_I$  phase as presumably occurs in the endosomal compartment upon acidification. In such a  $H_I$  phase, the plasmid DNA is intercalated between micelles where the polar head group of the amphiphile is exposed on the outside, giving rise to externally hydrophilic particles. In (b) the lamellar  $L_\alpha$  organization of SAINT-2/DOPE in the absence of salt and the inverted hexagonal  $H_{II}$  phase in its presence is displayed. In this  $H_{II}$  phase the polar head groups of the amphiphiles interact with the plasmid DNA and the hydrophobic tails are exposed on the outside, giving rise to externally hydrophobic particles that will tend to aggregate.

Fig. 6a shows a schematic representation of the phase transition that presumably occurs in the endosomes following internalization of the gemini lipoplexes. Upon acidification, the lamellar organization of the lipoplexes switches to a  $H_I$  phase. This type of hexagonal  $H_I$  phase at acidic pH, with DNA packed in between micelles, differs from the inverted hexagonal  $H_{II}$  phase described for SAINT-2/DOPE in salt (Fig. 6b). A mechanism based on solubilization of the membrane, as reported for detergents [25], can be envisioned for the destabilization of the endosomal membrane and the release of plasmid DNA. Indeed, our data (Fig. 5) showing extensive lipid mixing and DNA release, particularly at mild acidic pH, while simultaneously showing a strong dependence on the presence of PS, are entirely consistent with such a notion. This role of PS in the endosomal release of plasmid DNA has been described for other cationic lipids as well and likely requires the flip-flop of PS from the outer leaflet of the endosomal membrane to the inner leaflet [9,26]. It should be noted however, that strictly speaking the PicoGreen assay reports intercalation of the probe into accessible DNA. In previous studies we have shown by agarose gel analyses that DNA is actually released at these conditions [8]. In a micellar phase the lipid monomers are dynamic and will constantly transfer in and out of the micelles. Therefore, a competition can occur for the surfactant between binding to the DNA and interacting with the endosomal membrane, a process that likely includes translocation of endosomal lipids into the lipoplexes and which eventually leads to the release of DNA into the cytosol.

A potential of such a system for in vivo gene therapy applications can be envisioned since at physiological pH gemini lipoplexes have a lamellar organization, that will prolong their half time of circulation and hence improve their biodistribution by avoiding capture in the lung capillaries, as commonly seen for the  $H_{II}$ -forming lipoplexes [27,28]. Indeed, in a previous study we showed that following intravenous injection into mice, GS1 and GS2 lipoplexes did not lead to accumulation in and transfection of the lungs [17]. We propose that the application of these sugar-based gemini surfactants could be an alternative for the use of lipoplexes coated with PEG-lipids, necessary to stabilize and prevent aggregation of lipoplexes in the blood circulation.

## Abbreviations

DNA, deoxyribonucleic acid; lipoplexes, complexes of DNA with cationic lipids; DOPE or PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; DOTAP, *N*-[1-(2,3-dioleoyl)propyl]-*N,N,N*-trimethylammonium chloride; CTAB, cetyltrimethylammonium



bromide, EDLPC, ethyldilauroylphosphatidylcholine; EDOPC, ethyldioleoylphosphatidylcholine; SHN, sodium 3-hydroxy-2-naphthoate; SAINT-2, *N*-methyl-4-(dioleyl)methylpyridinium chloride;  $\lambda_{\text{max}}$  emission, maximum emission wavelength; SAXS, small angle X-ray scattering; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; HBS solution, HEPES buffered saline solution;  $L_{\alpha}$ , lamellar phase;  $L_I$ , micellar phase;  $H_{II}$ , inverted hexagonal phase;  $H_I$ , normal hexagonal phase

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